ORIGINAL ARTICLE: REPRODUCTIVE SCIENCE

Evaluation of ribonucleic acid amplification protocols for human oocyte transcriptome analysis

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Objective: To develop a reliable, reproducible, and sensitive method for investigating gene-expression profiles from individual human oocytes.
Design: Five commercially available protocols were investigated for their efficiency to amplify messenger RNA (mRNA) from 54 single human oocytes. Protocols resulting in sufficient yields were further validated using microarray technology. For the validation, mRNA was isolated from 25 human oocytes. To eliminate biological variation, RNA from 13 human oocytes was pooled together and split into 12 identical samples for further mRNA amplification. From 12 oocytes, mRNA was individually isolated.
Setting: University medical center and university microarray laboratory.
Patient(s): Couples undergoing intracytoplasmic sperm injection treatment were asked to donate their immature oocytes for research, and written informed consent was obtained in all cases. Seventy-nine human oocytes were used in total.
Intervention(s): None.
Main Outcome Measure(s): Amplification efficiency and microarray profiles.
Result(s): Two of the five protocols (WT-Ovation One-Direct and Arcturus RiboAMp HS Plus) resulted in sufficient yields and high success rates and were further validated for their performance in obtaining reliable, reproducible, and sensitive expression profiles from individual human oocytes. Evaluation of these two protocols demonstrated that they both displayed low technical variation and produced highly reproducible profiles ($r \geq 0.95$). One of them identified significantly more transcripts but also had a higher number of false discoveries.
Conclusion(s): Two protocols generated ample amounts of mRNA for (quantitative) polymerase chain reaction, microarray, and sequencing techniques. Further validation using a design that discriminates between biological and technical variation showed that both protocols can be used for gene-expression profiling of individual human oocytes. (Fertil Steril® 2015;■:■–■. ©2015 by American Society for Reproductive Medicine.)
Key Words: Gene expression, microarray, mRNA amplification, single human oocytes, validation

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Global gene-expression profiling using microarrays or next-generation sequencing has become an invaluable tool for the understanding of gene function and regulation. Until recently, large numbers of cells were required to obtain enough RNA for global gene-expression profiling. Although highly informative, this profile is often of limited value because it represents an average profile of all cells from the tissue under investigation, whereas the actual expression in any single cell most likely varies significantly (1–6). Furthermore, in some conditions, such as in studies investigating gametes or preimplantation embryos, only one or a few cells can be analyzed.

To overcome the inability to analyze single cells, many studies have pooled samples from the same experimental procedure to obtain sufficient RNA for microarray or sequencing analysis (7–13). Pooling is also often used to reduce the number of arrays or subject-to-subject variability (14, 15). Although pooling can be useful in some studies, it can

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introduce bias because the gene-expression profile derived from a pooled sample is not equivalent to the gene-expression profile of the individual samples (15). In addition, in some instances the experimental goal might be to study interindividual differences, which evidently precludes the use of pooling.

Recent developments now allow transcriptome analysis of single cells. Obtaining reproducible gene-expression profiles using microarray or sequencing technology in combination with limited starting material, such as single cells, requires amplification of the hybridization signal, mRNA amplification, or both. The methods aiming at intensifying fluorescence hybridization signal have significantly reduced the required RNA input but not to the level of a single cell (i.e., picogram amounts) (16). Additionally, there are many practical issues, like the difficulty in isolating and labeling low abundant transcripts (17). For messenger RNA (mRNA) amplification nowadays, several commercial kits are available.

Messenger RNA amplification protocols fall in two categories: those based on exponential and those based on linear amplification (18–21). The exponential-amplification polymerase chain reaction (PCR) protocols introduce PCR priming sites at both ends of each reversed-transcribed complementary DNA (cDNA) molecule, followed by global amplification via a number of PCR cycles. Most linear-amplification protocols use the Eberwine method, whereby double-stranded cDNA, generated by reverse transcription of mRNA and RNA polymerase utilizing a poly(T) oligonucleotide primer carrying a T7 promoter, undergoes in vitro transcription by T7 RNA polymerase. Alternatively, the Ribo-SPIA protocol uses a combination of chimeric DNA/RNA primers (3′ end and random primers), DNA polymerase, and RNase-H to linearly and isothermally amplify total RNA (22). For sequencing, another strategy has been applied to generate cDNA libraries, called rolling circle amplification. Here the RNA is reversed transcribed, circularized, and amplified using Phi29 DNA polymerase (23).

Polymerase chain reaction protocols are relatively simple and can easily amplify an RNA sample by approximately 1 million-fold in only a few hours. Limitations include the use of DNA polymerase having low efficiency in the amplification of GC-rich sequences and low fidelity leading to errors and their subsequent exponential amplification (reviewed in references 24 and 25). Additionally, the PCR reaction can lead to saturation if excess RNA input is used, favoring the amplification of high-abundant transcripts and distorting the original RNA distribution (25). Linear amplification does not disturb the original RNA distribution because the RNA polymerase is almost not affected by template sequences or the concentration of templates, and the reaction has a higher fidelity because errors do not accumulate exponentially (26). A single round produces up to a 1,000-fold amplification, which is not sufficient for most microarray- and sequencing-based protocols when subnanogram amounts are used. Therefore, additional amplification rounds are required that are time consuming and can lead to RNA degradation (25, 27–29). Optimized protocols for both PCR-based and linear amplification protocols have been described to overcome the previously mentioned disadvantages (30, 31).

However, irrespective of the protocol, there are common limitations associated with RNA amplification (reviewed in reference 24). For example, because most of these methods make use of the poly(A) tail of the mRNA, partially degraded RNA or noncoding RNA cannot be readily detected. Methods that use random primers together with oligo(dT) primers may overcome this problem, but then the primers should not be targeting the ribosomal RNA (rRNA) and transfer RNA (tRNA), or these RNA types should be depleted prior to amplification (32).

By analyzing single cells, the effect of biological difference(s) between cells can be measured. Technical replication is not possible because these samples have to be analyzed as a whole in one amplification step. Therefore, it is essential to find a protocol that can robustly and accurately amplify picograms input RNA from a single cell into micrograms necessary for further analysis. Additionally, the technique should exhibit high amplification efficiency and high signal enrichment and produce reproducible gene-expression patterns. Because the RNA content of individual cells can vary per tissue, it is important to validate the protocols directly on the cell type to be used in each study (33).

There are several successful strategies to reduce the required RNA input, and several studies have been published using few pooled or even single cells (7, 8, 11, 20, 33–39). However, the lower sensitivity limits have not adequately been validated with respect to accuracy and reliability of gene-expression analysis. Many studies reported on generated expression profiles from picograms of RNA. Some of these studies reported only on a small number of genes (1, 6, 40–43), whereas at the whole transcriptome level, studies have used populations of few cells (7–13, 33, 44), titrated dilutions of stock total RNA to picogram amounts, or single cells (3, 20, 36, 39, 45–54). Few studies, though, have validated the performance of whole transcriptome profiling from picogram amounts of input RNA (8–12, 20, 33–35, 38, 54–56), and only six, to our knowledge, actually used single cells (39, 45–47, 57, 58).

In this study we determined the technical and biological variability of samples, using commercially available single cell–based global gene-expression protocols to establish an optimal and robust workflow when working with single human oocytes.

MATERIALS AND METHODS

Biological Materials

Oocytes in the germinal vesicle stage were used for the technical validation. Couples undergoing intracytoplasmic sperm injection treatment at the Center for Reproductive Medicine of the Academic Medical Center of the University of Amsterdam (Amsterdam, the Netherlands) were asked to donate their immature oocytes for research, and written informed consent was obtained in all cases. Because spare immature human oocytes, which are normally discarded, were used, no ethical approval was deemed necessary by the central ethics committee on research involving human subjects. All couples